RESEARCH ARTICLE

SENP2 Regulates Hepatocellular Carcinoma Cell Growth by Modulating the Stability of β-catenin

Huo-Jian Shen, Hong-Yi Zhu, Chao Yang, Fu Ji*

Abstract

SUMOylation has emerged as an important post-translational modification that modulates the localization, stability and activity of a broad spectrum of proteins. A dynamic process, it can be reversed by a family of SUMO-specific proteases (SENPs). However, the biological roles of SENPs in mammalian development and pathogenesis remain largely elusive. Here, we demonstrated that SENP2 plays a critical role in the control of hepatocellular carcinoma cell growth. SENP2 was found to be down-regulated in hepatocellular carcinoma (HCC) tissues and over-expression suppressed the growth and colony formation of HCC cells. In contrast, silencing of SENP2 by siRNAs promoted cancer cell growth. We further found that stability of β-catenin was markedly decreased when SENP2 was over-expressed. Interestingly, the decrease was dependent on the de-SUMOylation activity of SENP2, because over-expression of a SENP2 catalytic mutant form had no obviously effects on β-catenin. Our results suggest that SENP2 might play a role in hepatocellular carcinoma cell growth control by modulating the stability of β-catenin.

Keywords: SENP2 - β-catenin - hepatocellular carcinoma - HepG2 cells

Asian Pacific J Cancer Prev, **13**, 3583-3587

Introduction

Small ubiquitin-like modifier (SUMO) was identified as a post-translational protein modifier over a decade (Geiss-Friedlander et al., 2007). SUMOylation affects the function of proteins by altering their localization, activity or stability (David, 2010). There are four members in the SUMO family, of which SUMO1–SUMO3 are ubiquitously expressed, whereas SUMO4 is mainly expressed in the lymph system. SUMO2 and SUMO3 are 97% identical, but share only 50% sequence identity with SUMO1 (Woo et al., 2010).

SUMOylation is a highly dynamic process which is catalyzed by SUMO-specific activating (E1), conjugating (E2), ligating (E3) enzymes and reversed SUMO-specific proteases (SENPs) (Bawa-Khalfe et al., 2010). There are two yeast SENPs, Ulp1 and Ulp2/Smt4, were first characterized (Li et al., 1999). Both the enzymes not only deconjugate SUMO/Smt3 from modified proteins but also process SUMO/Smt3 precursors to a mature form with a C-terminal diglycine. In mammalian cells, the SENP members were extent to six, based on their sequence homology, which can be divided into three subfamilies (Wang et al., 2009). SENP1 and SENP2 belong to the first sub-family and have broad substrate specificity. SENP1 is a nuclear protease that deconjugates a large number of SUMOylated proteins when over-expressed (Cheng et al., 2006). The mutation of SENP1 causes embryonic lethality due to a defective hypoxia-HIF1a pathway, which plays a crucial role in embryonic erythropoiesis(Cheng et al., 2007). SENP2 is a nuclear envelope-associated protease that, when over-expressed, appears to have an activity similar to that of SENP1 (Itahana et al., 2006). The second subfamily consists of SENP3 and SENP5, both of which are nucleolar proteins with preferences for SUMO2–SUMO3 (Di Bacco et al., 2006; Haindl et al., 2008). The third subfamily consists of SENP6 and SENP7, which have an extra loop in their catalytic domain(Vertegaal 2010). Although SENPs have shown activity in the SUMOylation process, specifically in deconjugation, their precise physiological role in reversing SUMOylation has not been well defined(Wang et al., 2009).

Previously, it was reported that SENP2 plays an essential role in embryonic development through modulating p53-Mdm2 pathway in placenta (Chiu et al., 2008). Whether SENP2 involves in tumorigenesis is completely unknown.

Here, we showed that SENP2 was down-regulated in hepatocellular carcinoma tissues. Over-expression of SENP2 inhibited hepatocellular carcinoma cells HepG2 growth while silencing of SNEP2 by siRNAs promoted the growth of HepG2 cells. Moreover, the stability of β-catenin was markedly decreased when SENP2 was over-expressed, and this effect was dependent on the de-SUMOylation activity of SENP2. Taken together, we found a potential role of SENP2 in hepatocellular carcinoma cell proliferation by modulating the stability of β-catenin.

*Department of General Surgery, Renji hospital of Shanghai Jiao Tong University School of Medicine, Shanghai, China *For correspondence: jifu_renji@hotmail.com*

Materials and Methods

Tissue samples

Tumor tissues and adjacent normal tissues from 25 cases of hepatocellular carcinoma patients were collected from Renji hospital of Shanghai Jiao Tong University School of Medicine. All samples were obtained with informed consent and approved by Renji hospital of Shanghai Jiao Tong University School of Medicine clinic institutional review board.

Cell culture and regents

Hepatocellular carcinoma cell line HepG2 (from Cell Bank of Shanghai Institutes of Biological Sciences, Shanghai, China) was cultured in RPMI-1640 medium (Sigma, St Louis, MI) supplemented with 10% fetal calf serum and cultured in a 5% CO2/95% air at 37°C. DMSO and drugs in this work were purchased from sigma.

Antibodies

Antibodies were obtained from the following sources: anti-SENP2 (Santa Cruz Biotech, Santa Cruz, CA), anti-C-Myc (Cell Signaling, Beverly, MA), anti-Flag M2 (Sigma, St Louis, MO), anti-β-catenin (Santa Cruz Biotech, Santa Cruz, CA) and anti-β-actin (Cell Signaling, Beverly, MA), Plasmids and Transfection

All the plasmids of this work are purchased from Addgene. All the transient transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

siRNAs

All of the lentiviral siRNA vectors were purchased from Santa cruz.

Colony formation assay

Each 35 mm culture dish contained a base layer consisting of 0.5 ml culture media (0.6% agar). Cells were cultured by layering 5000 cells in 0.5 ml culture media (0.3% agar) over each base layer. All cells formed sufficient numbers of detectable colonies following 14 days incubation.

RNA isolation and Real-time PCR analysis

Total RNA was isolated from tissues or cells using TRIzol (Invitrogen) according to the manufacturer's instructions. In order to quantify the transcripts of the interest genes, Real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on LightCycler 480 (Roche, Switzerland). The primers used were available upon request.

Western blotting

Protein extracts were equally loaded on 10% SDS–PAGE, electrophoresed, and transferred to nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK). After blocking with 5% nonfat milk in PBS, the membranes were incubated with the indicated primary antibodies and followed by horseradish peroxidase (HRP)–linked secondary antibodies (Cell signaling). The signals were detected by chemiluminescence phototope- HRP kit (Pierce Biotechnology, Rockford, USA) according to manufacturer's instructions.

Statistical Analysis

Values were shown as mean±SD. Statistical differences were determined by a Student's t test. Statistical significance is displayed as * ($P < 0.05$) ,** ($P < 0.01$) or ***($P < 0.001$).

Results

SENP2 was down-regulated in hepatocellular carcinoma tissues and cells

To investigate the expression of SENP2 in hepatocellular carcinoma, we detected the mRNA level of SENP2 in tumors and their adjacent normal tissues from 25 primary hepatocellular carcinoma samples by real-time quantitative PCR. The relative folds of SENP2 mRNA of hepatocellular carcinoma tissues against their adjacent tissues were calculated. As shown in Figure 1A, the SENP2 mRNA was down-regulated in 18 out of total 25 cancer tissues when compared with the paired adjacent normal tissues. We also checked the protein expression of SENP2 in those clinical tissues including 5 hepatocellular carcinoma tissues and the paired adjacent normal tissues by western blot with SENP2 antibody and found that SENP2 was indeed down-regulated in hepatocellular carcinoma tissues (Figure 1B).

Ectopic expression of SENP2 suppressed the growth and colony formation of HepG2 cells

The fact that SENP2 is down-regulated in hepatocellular carcinoma tissues raised a question that whether SENP2 played a role in the pathogenesis of hepatocellular carcinoma. Hence, we ectopic expressed Flag-SENP2 in

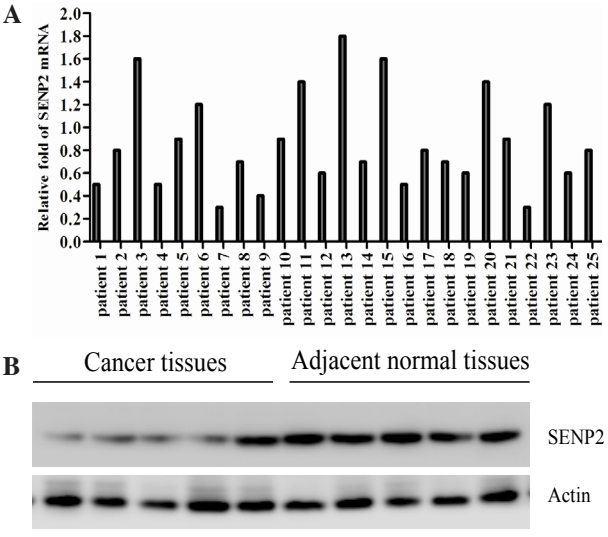

Figure 1. SENP2 was Down-regulated in Gastric Cancer Tissues. A. The mRNA levels of SENP2 in tumor tissues and their adjacent normal tissues of 25 hepatocellular carcinoma patients were detected by real-time quantitative PCR, the relative mRNA expression levels of SENP2 in tumor tissues against that of paired adjacent normal tissues were shown. B. The protein levels of SENP2 in tumor tissues and their adjacent normal tissues of 5 hepatocellular carcinoma patients were detected by western blot with SENP2 antibody

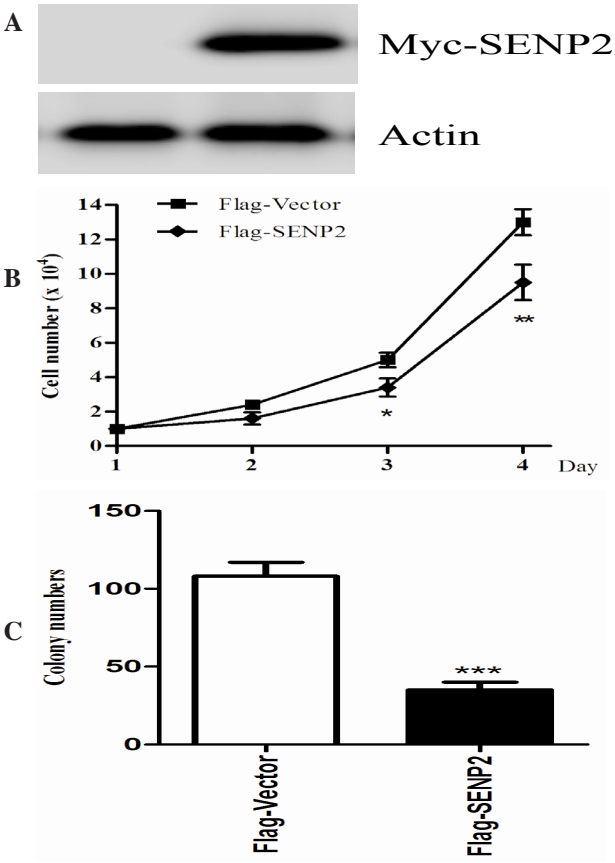

Figure 2. Ectopic Expression of SENP2 Suppressed the Growth and Colony Formation of HepG2 Cells. A. The expression of exogenous SENP2 in HepG2 cells was detected by western blot with Flag antibody. B. Growth curves of Flag-con- HepG2 and Flag-SENP2-HepG2 cells. C. Numbers of colony of Flag-con-HepG2 and Flag-SENP2-HepG2 cells

HepG2 cells by retrovirus mediated gene delivery. The exogenous expression of SENP2 in HepG2 cells was confirmed by western blot with Flag antibody (Figure 2A). Over-expression of SENP2 significantly decreased the growth of HepG2 cells (Figure 2B). Consistent with this, the number of colony formed in soft-agar was significantly decreased when SENP2 was over-expressed (Figure 2C).

Silencing of SENP2 by siRNAs promoted growth and colony formation of HepG2 cells

Since over-expression of SENP2 suppressed the growth of HepG2 cells, we next asked whether knockdown of SENP2 in HepG2 cells affected its growth. To rule it out, we silenced SENP2 in HepG2 cells by introducing two different siRNAs and both siRNAs suppressed the expression of endogenous SENP2, although number 2 achieved a more significant knockdown efficiency when compared with number 1 siRNA (Figure 3A). As predicted, silencing of SENP2 significantly promoted the growth (Figure 3B) and colony formation (Figure 3C) of HepG2 cells

Over-expression of SENP2 decreased the expression of β-catenin

To address how SENP2 affected the growth of HepG2 cells, we tested the possible effects of SENP2 on the expression of tumor related genes, including β-catenin and C-myc. The results showed that over-expression of SENP2

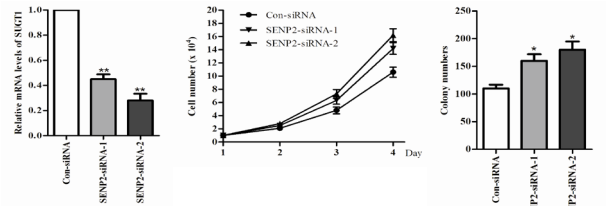

Figure 3. Silencing of SENP2 Expression Promoted the Growth and Colony Formation of HepG2 Cells. A. Relative mRNA expression levels of SENP2 were measured by real-time PCR in si-con-HepG2 cells and si-SENP2-HepG2 cells. B. Growth curves of si-con-HepG2 cells and si-SENP2-HepG2 cells. C. Numbers of colony of si-con-HepG2 cells and si-SENP2-HepG2 cells

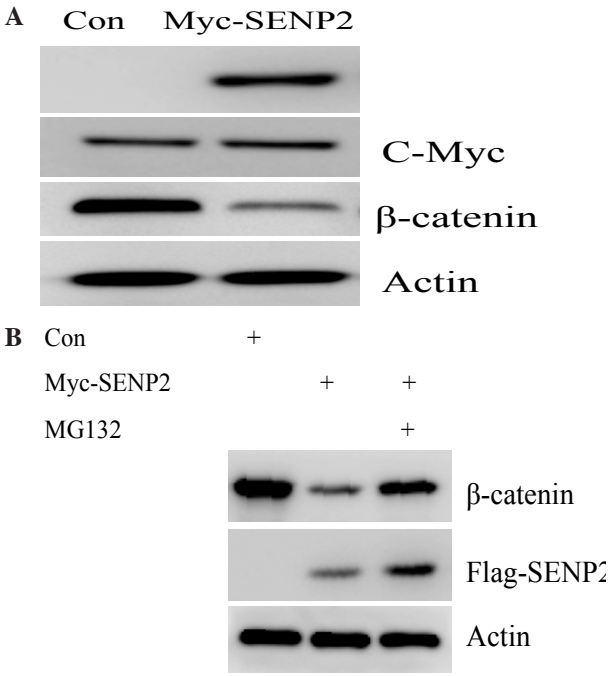

Figure 4. Over-expression of SENP2 Promoted the Degradation of β-catenin. A. Relative protein levels for C-Myc and beta-catenin were determined in HepG2 cells with or without Flag-SENP2. B. HepG2 cells transfected with indicated plasmids and treated with MG132 (20μM) for 6 h where indicated before harvested, the protein levels of beta-catenin were measured by western blot

did not affect the expression of C-myc in HepG2 cells, but significantly decreased the expression of β-catenin (Figure 4A). The decreased expression of β-catenin could be partially rescued by the proteasome inhibitor MG132 (Figure 4B), suggesting that over-expression of SENP2 promoted the degradation of β-catenin,

Silencing of SENP2 by siRNAs increased the expression of β-catenin

To further confirm that β-catenin was regulated by SENP2, we also checked the protein levels of β-catenin in SENP2 silencing cells. Indeed, the protein level of β-catenin was increased when SENP2 was silenced (Figure 5).

SENP2 regulated expression of β-catenin depended on its catalytic activity

The known biological function of SENP2 was documented to be dependent on its catalytic activity.

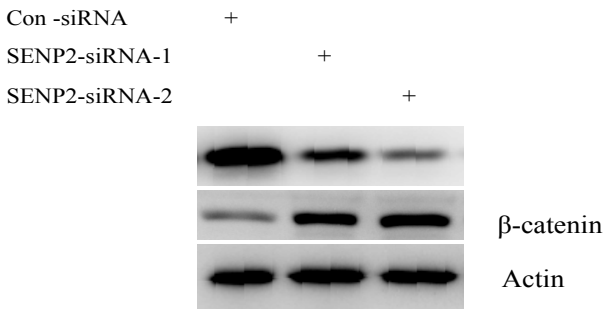

Figure 5. Silencing of SENP2 by siRNAs Increased the Expression of β-catenin. A. Relative protein levels for C-Myc and beta-catenin were determined in si-con-HepG2 cells and si-SENP2- HepG2 cells

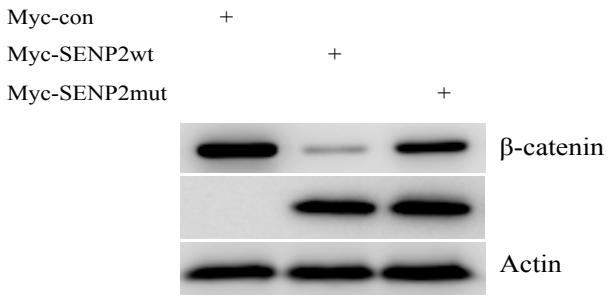

Figure 6. SENP2 Regulated Expression of β-catenin Depends on Its Catalytic Activity. A. HepG2 cells transfected with either SENP2 WT or SENP2 Mut plasmids for 36h, western blot was performed with the indicated antibodies

To address whether SENP2 regulated expression of β-catenin depended on its catalytic activity, we over-expressed SENP2 wild type (SENP2 WT) and SENP2 catalytic activity mutant form (SENP2 Mut) in HepG2 cells. Consistent with the above data, over-expression of SENP2 WT decreased the expression of β-catenin (Figure 6). In contrast to SENP2 WT, over-expression of SENP2 Mut had little effects on the expression of β-catenin protein level (Figure 6). These data highlighted the de-SUMOylation activity of SENP2 in the regulation of β-catenin expression.

Discussion

Wnt signaling is one of the most critical pathways for regulation of cell proliferation, differentiation and migration during embryonic patterning and morphogenesis (Huang et al., 2008). One of the key events of canonical or Wnt/β-catenin-dependent pathways is accumulation and nuclear translocation of β-catenin, which functions as a transcriptional co-activator of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA-binding transcription factors (MacDonald et al., 2009). Dysregulation and aberrant activation of Wnt pathways or mutations in β-catenin or adenomatous polyposis coli (APC) often results in increased β-catenin accumulation (Lam et al., 2011). The oncogenic potential of nuclear β-catenin in the initiation and progression of various human malignancies including hepatocellular carcinoma have been investigated (Cavard et al., 2008; Zhang et al., 2010; Anson et al., 2012; White et al., 2012)

SENP2 was originally reported to be associated with the nuclear envelope through binding to Nup153(Hang et al., 2002). Recently study suggested that SENP2 is the key regulator of Pc2/CBX4 function through regulation of the SUMOylation status of Pc2/CBX4 (Kang et al., 2010). Disruption of SENP2 leads to developmental defects in trophoblast stem cell niches and lineages through modulating p53-Mdm2 pathway in placenta (Chiu et al., 2008). Given the facts that p53 is frequently inactive in many cancers and aberrant activate of embryonic signaling usually leading to cancer development, it is will be not surprised that SENP2 may play a role in carcinogenesis.

Here, we described another way that regulated the expression of β-catenin by SENP2 in hepatocellular carcinoma cells. First, we found that SENP2 was down-regualted in most hepatocellular carcinoma tissues we tested. This down-regulation came with rapidly growth of hepatocellular carcinoma cells, as inhibition the expression of SENP2 by two different siRNAs increased the growth speed of HepG2 cells. Moreover, Over-expression of SENP2 inhibited the expression and accelerated the degradation of β-catenin, whereas suppression of SENP2 caused increased expression of β-catenin, indicating dyregulated Wnt pathway may responsible for the biological functions of SENP2. Interestingly, the de-SUMOylation activity of SENP2 was required for it regulated β-catenin expression, indicating an even more complicated mechanism in this regulation cascades. However, the direct target of SENP2 that may regulate the stability of β-catenin is still not known, further work is required to identify the direct target of SENP2 in hepatocellular carcinoma cells.

In conclusion, our data supported the idea that SENP2 controlled hepatocellular carcinoma growth through the regulation of the stability of β-catenin.

Acknowledgements

The author(s) declare that they have no competing interests.

References

Anson M, Crain-Denoyelle AM, Baud V, et al (2012). Oncogenic beta-catenin triggers an inflammatory response that determines the aggressiveness of hepatocellular carcinoma in mice. *J Clin Invest,* **122**, 586-99.

Bawa-Khalfe T, Yeh ET (2010). SUMO Losing Balance: SUMO Proteases Disrupt SUMO Homeostasis to Facilitate Cancer Development and Progression. *Genes Cancer,* **1**, 748-52.

Cavard, C, S Colnot, V Audard, et al (2008). Wnt/beta-catenin pathway in hepatocellular carcinoma pathogenesis and liver physiology. *Future Oncol,* **4**, 647-60.

Cheng J, Bawa T, Lee P, et al (2006). Role of desumoylation in the development of prostate cancer. *Neoplasia,* **8**, 667-76.

Cheng J, Kang X, Zhang S, et al (2007). SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell,* **131**, 584-95.

Chiu SY, Asai N, Costantini F, et al (2008). SUMO-specific protease 2 is essential for modulating p53-Mdm2 in development of trophoblast stem cell niches and lineages. *PLoS Biol,* **6**, e310.

David R (2010). Sumoylation: Targeting SUMO. *Nat Rev Mol Cell Biol,* **11**, 387.

Di Bacco A, Gill G (2006). SUMO-specific proteases and the

cell cycle. An essential role for SENP5 in cell proliferation. *Cell Cycle,* **5**, 2310-3.
Geiss-Friedlander R, Melchior F (2007). Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol,* **8**, 947-56.
Haindl M, Harasim T, Eick T, et al (2008). The nucleolar SUMO-specific protease SENP3 reverses SUMO modification of nucleophosmin and is required for rRNA processing. *EMBO Rep,* **9**, 273-9.
Hang J, Dasso M (2002). Association of the human SUMO-1 protease SENP2 with the nuclear pore. *J Biol Chem,* **277**, 19961-6.
Huang H, He X (2008). Wnt/beta-catenin signaling: new (and old) players and new insights. *Curr Opin Cell Biol,* **20**, 119-25.
Itahana Y, Yeh ET, Zhang Y (2006). Nucleocytoplasmic shuttling modulates activity and ubiquitination-dependent turnover of SUMO-specific protease 2. *Mol Cell Biol,* **26**, 4675-89.
Kang X, Qi Y, Zuo Y, et al (2010). SUMO-specific protease 2 is essential for suppression of polycomb group protein-mediated gene silencing during embryonic development. *Mol Cell,* **38**, 191-201.
Lam AP, Gottardi CJ (2011). Beta-catenin signaling: a novel mediator of fibrosis and potential therapeutic target. *Curr Opin Rheumatol,* **23**, 562-7.
Li SJ, Hochstrasser M (1999). A new protease required for cell-cycle progression in yeast. *Nature,* **398**, 246-51.
MacDonald BT, Tamai K, He X (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell,* **17**, 9-26.
Vertegaal AC (2010). SUMO chains: polymeric signals. *Biochem Soc Trans,* **38**, 46-49.
Wang Y, Dasso M (2009). SUMOylation and deSUMOylation at a glance. *J Cell Sci,* **122**, 4249-52.
White BD, Chien AJ, Dawson DW (2012). Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. *Gastroenterology,* **142**, 219-32.
Woo CH, Abe J (2010). SUMO--a post-translational modification with therapeutic potential? *Curr Opin Pharmacol,* **10**, 146-55.
Zhang XF, Tan X, Zeng G, et al (2010). Conditional beta-catenin loss in mice promotes chemical hepatocarcinogenesis: role of oxidative stress and platelet-derived growth factor receptor alpha/phosphoinositide 3-kinase signaling. *Hepatology,* **52**, 954-65.